Absorbed EVELISA: A Diagnostic Test with Improved Specificity for Johne’s Disease in Cattle

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Abstract

The use of enzyme-linked immunosorbent assays (ELISAs) is recommended for Johne’s disease (JD) control in dairy herds. In 2006, we developed a novel ELISA test for JD, named EVELISA (ELISA using ethanol extract of Mycobacterium avium subsp. paratuberculosis), which showed higher sensitivity than commercial ELISA tests. To further investigate the performance of EVELISA, we obtained 38 serum samples from cattle in a JD-free herd with suspected cases of serological false-positive reactions. When these samples were tested using the EVELISA and a commercial ELISA test, more than 70% of the samples were falsely identified as JD positive. Antibodies in the serum samples reacted strongly with antigens of various environmental mycobacteria, suggesting the presence of cross-reactive antibodies in the samples. The possible cross reactions in the EVELISA were inhibited markedly by the use of Mycobacterium phlei antigens for antibody absorption. When these samples were tested, 8 samples were classified as positive for JD by the EVELISA with the antibody absorption, whereas 27 samples were classified as positive for JD by the commercial ELISA. For an estimation of tentative sensitivity and specificity, the ELISA tests were performed on 38 serum samples from JD-negative herds with no suspected cases of serological false-positive reaction and 68 samples from cattle diagnosed as positive for M. avium subsp. paratuberculosis infection by fecal culture test. Sensitivity and specificity of the EVELISA with preabsorption of serum with M. phlei (“ethanol vortex absorbed-ELISA” or EVA-ELISA) were estimated to be 97.1% and 100%, respectively, whereas those of the commercial ELISA were 48.5% and 97.4%, respectively. Further, in 85 fecal culture-negative cattle in JD-positive herds, higher sensitivity of the EVA-ELISA than the commercial ELISA was demonstrated by a Bayesian analysis. This study indicates that the EVA-ELISA may form a basis for a sensitive diagnostic test with a higher level of specificity than that of the current commercial ELISA test.

Introduction

JOHNE’S DISEASE (JD) is a chronic, granulomatous enteritis of ruminants caused by an environmental mycobacterium, Mycobacterium avium subsp. paratuberculosis (MAP) (Cocito et al., 1994; Stabel, 1998). Mainly because of the reduction of milk production and premature culling of affected cattle, JD causes significant ongoing economic losses in the dairy industry (Hendrick et al., 2005; Tiwari et al., 2008). Complicating factors associated with JD control are the relatively low accuracy of current enzyme-linked immunosorbent assays (ELISAs) for the disease. For instance, a Bayesian statistical analysis of nongold standard data by Wells et al. (2006) determined that the estimated sensitivities of current ELISAs for JD were 26%–27%. In addition to the low sensitivity issue, several reports indicated that environmental mycobacteria caused false-positive reactions in current ELISA tests. For example, Roussel et al. (2007) reported a positive correlation between recovery of environmental, non-MAP mycobacteria from feces and false-positive ELISA results, which indicates that infection of cattle with environmental mycobacteria is linked to false-positive reactions in current ELISAs for JD.

We previously developed novel serological diagnostic tests for JD that showed higher sensitivities than commercial ELISA tests (Eda et al., 2005, 2006; Speer et al., 2006). As these tests did not cross-react with sera from artificially infected

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cattle with Mycobacterium avium subsp. avium (MAA) or Mycobacterium bovis, we hypothesized that the tests had higher specificity (lower false-positive rate) than commercial ELISA tests. In this study, one of those tests, named EVELISA (ELISA using ethanol extract of MAP) (Eda et al., 2006), and a commercially available ELISA were tested for their specificity using bovine serum samples obtained from a JD-free herd with suspected cases of serological false-positive reactions. Also, serum samples from dairy herds with no reported suspected cases of false-positive reactions were tested to estimate and compare tentative sensitivities and specificities of the EVELISA and the commercial test.

Materials and Methods

ELISA procedures

Commercial ELISA. A commercial ELISA (IDEXX Laboratories, Westbrook, ME), termed ELISA-A in this study, was carried out according to the manufacturer’s instructions. Also, according to the instructions, the ratio of optical densities of sample and positive control (S/P value) was determined and JD status was assigned using a cutoff S/P value of 0.25.

In-house ELISA. Another ELISA that we developed using ethanol extract of MAP, named EVELISA, was conducted as described previously (Eda et al., 2006). MAP strains (Linda and K10) used for the EVELISA antigen preparation were obtained from the Agricultural Research Service, U.S. Department of Agriculture (Ames, Iowa). Other environmental mycobacteria were used for testing cross reactions of serum antibodies. The environmental mycobacteria included MAA, Mycobacterium scrofulaceum, Mycobacterium szulgai, Mycobacterium gordonae, Mycobacterium kansasi, M. bovis Bacillus Calmette-Guérin (BCG) and Mycobacterium phlei (provided by Dr. P. Small, Department of Microbiology, The University of Tennessee, Knoxville). Mycobacteria were cultured in Middlebrook’s medium (Becton Dickinson, Cockeysville, MD) supplemented with 0.05% Tween 80 (Fisher Scientific, Fair Lawn, NJ) at 37°C until used for experimentation. In the case of MAP, 2 mg/L Mycobactin J (Allied Monitor, Fayette, MO) was added to the culture medium. These Mycobacterium species were originally obtained from the American Type Culture Collection. Thus, they have been authenticated by the depositors as well as the curator. Mycobacteria were harvested from the liquid culture, suspended in 80% ethanol, and agitated by vortex to dislodge surface antigens. After removing the bacilli by centrifugation, dislodged antigen in the ethanol solution was immobilized on a 96-well plate by evaporating the solution, reacted with diluted serum samples followed by horseradish peroxidase-labeled goat anti-bovine immunoglobulin G (H + L) polyclonal antibody (Jackson ImmunoResearch Lab, Westgroun, PA). Binding of bovine serum antibody was detected by using a substrate of horseradish peroxidase (2,2’-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) according to the manufacturer’s instructions. For the EVELISA, S/P values were calculated and a cutoff S/P value of 0.20 was determined as described previously (Eda et al., 2006). Pre-absorption of cross-reactive antibodies in serum samples was conducted by diluting the samples 100× with buffer A (10 mM phosphate-buffered saline, pH 7.0, containing 0.05% [v/v] Tween 80 and 10% [v/v] SuperBlock [Pierce Biotechnology, Rockford, IL]) containing 0.5 mg/mL M. phlei Absorben (Allied Monitor) and incubating at room temperature for 30 min.

Serum samples

Group I. Serum samples were obtained from 38 Holstein cattle (aged 2–9 years [mean ± standard deviation = 5.18 ± 2.01]) in a Japanese dairy herd (total head count: 147) with no history of JD. JD-negative status of the herd was confirmed by a whole-herd testing procedure that involved repeated applications of fecal culture and real-time polymerase chain reaction (PCR) tests as follows. Prior to serum sampling, all animals in the herd were diagnosed as negative for MAP infection by the PCR test for three times with 2–4-month intervals. Also, all the animals in the herd were diagnosed as negative for MAP infection by the fecal culture test for six times with 5–15-month intervals. No JD-characteristic clinical sign was observed in animals in the herd. However, false-positive reactions in serum samples of several cattle were indicated by ELISA and complement fixation tests. The 38 cattle were selected to include most of the EVELISA-positive animals and serum samples of the animals were collected at the same time of the last fecal culture test. Fecal culture tests were done using Herrold’s egg yolk medium with Mycobactin at 2 mg/L after treatment of fecal samples with 0.75% hexadecylpyridinium chloride. Serum antibodies were detected by commercial ELISA and complement fixation tests with antigen produced at the biological products section of the National Institute of Animal Health. A real-time PCR test of fecal DNA samples was performed using the same methods described by Kawaji et al. (2007).

Group II. To evaluate the specificity of the ELISA tests under investigation here, 38 serum samples were obtained from cattle (female Holstein-Friesian cattle, aged 0.5–9 years [mean ± standard deviation = 3.4 ± 2.7]) in two Japanese herds; all 38 of these samples were negative for JD by fecal culture test. The fecal culture test was carried out once prior to the serum sample collection. No JD-characteristic clinical sign was observed in animals in the herds. Further, all the animals were diagnosed as negative for JD by a commercial ELISA (Kyoritsu Seiyaku, Tokyo, Japan).

Group III. To evaluate the sensitivity of the ELISA tests used in this study, 68 serum samples were obtained from female Holstein-Friesian cattle in three dairy herds in Pennsylvania and Minnesota (aged 1–8 years [mean ± standard deviation = 4.1 ± 2.0]). These cattle were diagnosed as positive for MAP infection by the fecal culture test. As these 68 cattle had repeatedly (at least twice) diagnosed as positive for MAP infection by fecal culture tests that were conducted at 6-month intervals in a longitudinal study (Whitlock et al., 2000), it is unlikely that these are false positives due to “pass through” of bacilli (i.e., positives due to organisms passed through the digestive tract without causing infection). Of the 68 cattle, 36, 2, and 30 animals were categorized as low, medium, and high shedders, respectively, according to the criteria used in a previous study (Whitlock et al., 2000).

Group IV. Eighty-five serum samples were collected from cattle on the same farms as Group III. These animals were...
diagnosed as negative for MAP infection by the fecal culture test. These samples were used for Bayesian estimation of the probability sensitivity of a modified EVELISA.

In addition to these grouped samples, we used three serum samples collected from female Holstein-Friesian cattle in another Japanese herd. The animals were diagnosed as positive for MAP infection by PCR and fecal culture tests, and the serum samples were called “PCR- and fecal culture-positive (PF-P) serum samples” in this study. These samples were used to examine antibody binding to antigens of various mycobacteria and to evaluate the effect of different concentrations of M. phlei on antibody binding in EVELISA.

Statistical analysis

A Bayesian analysis of data from fecal culture-negative samples from JD-positive herds was conducted using a one-population binomial model (Branscum et al., 2004). The model was applied to ELISA data (test positive or test negative) from cattle with uncertain MAP infection status. The probability of a positive test (apparent prevalence) was $AP = \pi Se + \{1 - \pi\}(1 - Sp)$. From this equation, the sensitivity of the ethanol vortex absorbed-ELISA (EVA-ELISA) test is given by the following equation: $Se = [AP - \{1 - \pi\}(1 - Sp)]/\pi$. The data were obtained from only fecal culture-negative cattle, so $\pi = Pr[JD \text{ positive} \mid \text{fecal culture negative}]$. Using Bayes’ theorem, $\pi = (1 - Se_{FC})p/(1 - Se_{FC})p + Sp_{FC}(1 - p)$, where $Se_{FC}$ and $Sp_{FC}$ are the sensitivity and specificity of the fecal culture test, respectively, and $p$ is the fraction of animals that have JD within a JD-positive source population. The posterior distribution of $Se$ was approximated using a Monte Carlo sample of 50,000 iterates after a burn-in of 10,000 was discarded. The posterior probability that $Se$ exceeds a value $c$ is calculated using the following equation: $m^{-1}\sum_{j=1}^{m} I_c(Se^j)$, where $m = 50,000$, $Se^j$ denotes the $j$th simulated iterate from the posterior distribution of $Se$, and $I_c(x)$ denotes the indicator function, which equals 1 if $x \in A$ and 0 otherwise.

The nongold standard analysis contained parameters that were not estimable from the data alone, so additional input was needed to obtain inferences. This input manifested in the form of informative prior distributions, which were constructed using information from previously published research (Branscum et al., 2004, 2005; Behr and Kapur, 2008; Mc et al., 2008). WinBUGS version 1.4.3 (Lunn et al., 2000) was used for Bayesian data analysis.

Results

Among the 38 Group I samples (sera from cattle in a JD-free herd with suspected cases of serological false-positive reactions), the ELISA-A and EVELISA incorrectly classified 72.9% and 75.7% of the samples as positive for JD, respectively.

Two Group I serum samples and two PF-P serum samples were tested for antibody binding to antigens of 10 different mycobacterial species. The levels of antibody binding to MAP extracts in the Group I samples were almost equivalent to those to other mycobacteria except M. bovis BCG and M. szulgai (Fig. 1A). In contrast, the levels of antibody binding to MAP extracts in the two PF-P serum samples were greater than those to extracts of other mycobacteria species (Fig. 1B). The antibody in the Group I samples reacted most strongly with M. phlei extract (Fig. 1A). Therefore, M. phlei was used in the following experiments as an absorbent.

Three Group I samples and three PF-P serum samples were preabsorbed with 0–2 mg/mL of M. phlei and tested for reactivity by the EVELISA. Antibody binding in the Group I samples was strongly inhibited by the M. phlei preabsorption, whereas that in the three PF-P samples was not decreased by preabsorption with M. phlei even at the highest concentration tested (Fig. 2). Further, we tested 33 Group I samples and 66 Group III samples with or without M. phlei (0.5 mg/mL) preabsorption. The percent of inhibition (average) caused by M. phlei preabsorption was 96.8% in Group I and 28.8% in Group III. The EVELISA with preabsorption

FIG. 1. Serum antibody reactions against various mycobacteria. Serum samples from two animals in Group I (animal 1 [open bar] and animal 2 [hatched bar]) (A) and samples from two polymerase chain reaction- and fecal culture-positive animals (animal 3 [open bar] and animal 4 [hatched bar]) (B) were reacted with antigens of various mycobacteria by the EVELISA procedure. Each bar represents mean ± standard deviation of antibody binding. Mycobacterium species used for the antigen preparation: 1, MAP Linda strain; 2, MAP 1974 strain; 3, Mycobacterium avium subsp. avium; 4, M. scrofulaceum; 5, M. bovis BCG; 6, M. szulgai; 7, M. gordone; 8, M. kansasii; 9, M. phlei; 10, no antigen. EVELISA, ELISA using ethanol extract of MAP; MAP, Mycobacterium avium subsp. paratuberculosis.
of serum with *M. phlei* at a concentration of 0.5 mg/mL was named EVA-ELISA.

When 38 Group I samples were tested, only 8 (21.1%) were classified as positive for JD by EVA-ELISA, whereas 27 (71.1%) were classified as positive by ELISA-A (Fig. 3). Using 38 Group II (sera from cattle in JD-negative herds) and 68 Group III (sera from fecal culture-positive animals) samples, empirical sensitivity and specificity of the EVA-ELISA at a cutoff value of 0.20 were 97.1% and 100%, respectively (Fig. 3A), whereas those of ELISA-A were 48.5% and 97.4%, respectively (Fig. 3B).

Although the primary aim of the current study was to evaluate and compare false-positive rates, in a secondary analysis we tested whether the sensitivity of EVA-ELISA was higher than that of ELISA-A in a particular subgroup (Group IV, fecal culture-negative cattle). The sensitivities in Fig. 3A apply to fecal culture-positive cattle, which generally exclude newly and subclinically infected animals. Therefore, we also conducted an analysis of 85 Group IV samples, of which 14 were classified as positive for JD by EVA-ELISA. We set SpFC = 1 and used values of SeFC and *p* that have appeared in previously published JD studies, that is, SeFC = 0.60 (Messam et al., 2008) and *p* = 0.12 (Branscum et al., 2004). These inputs yield *n* = 0.052. On the basis of Group II data, which showed perfect specificity of EVA-ELISA, we set Sp = 1. The plug-in estimate of Se, which uses the unbiased estimate of AP of 0.165 (= 14/85), exceeds 1. A Bayesian analysis yields a range-respecting estimate of Se when a uniform (0, 1) prior distribution is used for this parameter. With this prior, we calculated the posterior probability that Se > 0.485, where 48.5% is the sensitivity of ELISA-A calculated using Group III data. As Group III involved fecal culture-positive animals only, we did
not expect the sensitivity of ELISA-A to exceed 48.5% when applied to fecal culture-negative samples. Therefore, we calculated the posterior probability of the hypothesis $H$: $Se_{EVA}$ to be 0.485 to investigate whether the $Se_{EVA}$ of EVA-ELISA among fecal culture-negative cattle (Group IV) exceeded the expected maximum sensitivity of ELISA-A in this group. The posterior probability of $H$ is 1. This result was virtually unchanged when we modeled $p$ by placing a beta prior on $p$ (instead of fixing the value of $p$ at 0.12) that had a mode of 0.12 and 95% prior interval (0.04, 0.33). The exact prior is $p \sim \text{beta}(3.28, 17.74)$, which has been previously used as a prevalence prior for JD-positive herds (Branscum et al., 2004). The same posterior probability also results from a prior on $Se_{EVA}$ that is based on Group III data. To assess the impact of uncertainty about the empirical sensitivity of ELISA-A (48.5%), we calculated the posterior probability that $Se_{EVA}$ exceeds the much higher value of 70%. The probability of 0.99 indicates a high degree of robustness to the presumed value of 48.5% for the sensitivity of ELISA-A.

**Discussion**

ELISA test continues to be used as one of the major diagnostic procedures to support the control of JD; however, the current commercially available ELISA tests suffer low sensitivity (Whitlock et al., 2000; Nielsen and Toft, 2008) and are subject to low specificity (Osterstock et al., 2007; Roussel et al., 2007). Indeed, in this study, we found that a commercial ELISA (ELISA-A) and our in-house ELISA (EVELISA) had low specificity in sera of cattle from a JD-free dairy herd with suspected cases of serological false-positive reaction. However, the possible false-positive reactions in the EVELISA were subsequently reduced markedly with preabsorption of cross-reactive antibodies using $M.\ phlei$ antigens (EVA-ELISA) without sacrificing the sensitivity of the EVELISA test. A large-scale, longitudinal prospective study is required to validate the EVA-ELISA’s sensitivity and specificity estimated in this study.

Previous large-scale studies showed that specificities of commercially available ELISAs were high (e.g., 97%) (Nielsen and Toft, 2008), indicating that the false-positive reactions of ELISAs are not a widespread issue in dairy herds. We, therefore, did not use the data obtained from Group I samples for estimation of specificity. Roussel et al. (2007) reported that five beef herds in a southeastern region of Texas had high (22%–45%) seroprevalence of JD with low rates of MAP isolation (2%–7%), which could indicate a low specificity of the ELISA in the region (Roussel et al., 2007). In this study, the specificity of EVA-ELISA was almost three times higher than ELISA-A in a dairy herd with suspected cases of serological false-positive reactions. Therefore, it will be of interest to examine how EVA-ELISA performs in the beef herds where current ELISAs showed low specificity.

Roussel et al. (2007) also showed that environmental mycobacteria were isolated from feces of 9%–43% of the cattle in the high-seroprevalence herds, whereas the rate was much lower (4%–6%) in cattle in geographically matched herds, suggesting that the possible low specificity of ELISA-A was due to the infection of cattle with environmental mycobacteria. Indeed, the commercial ELISA used in their study cross-reacted with antibodies in sera of calves experimentally infected with MAA, *Mycobacterium intracellulare*, *M. scrofulaceum*, or *Mycobacterium terrae* (Osterstock et al., 2007). Therefore, the high proportion of antibody reactions in Group I samples in EVELISA may also have been due to infections of cattle with environmental mycobacteria. As MAA and *M. scrofulaceum* have been isolated from soil and water in cattle herds (Donoghue et al., 1997; Norby et al., 2007), these species may have been the cause of false-positive reactions in the EVELISA test.

Since Yokomizo et al. (1983, 1985) determined that heat-killed *M. phlei* was effective in reducing false-positive reactions in ELISA tests for JD, the bacteria has been routinely used for preabsorption of cross-reactive antibodies in commercial ELISAs. In this study, *M. phlei* antigens cross-reacted most strongly with two Group I samples tested. The ELISA-A test had strong reactions in Group I samples, despite that it uses *M. phlei* for preabsorption of cross-reactive antibodies. In contrast, antibody reactions of Group I samples in EVELISA were substantially decreased upon using *M. phlei*. Strong inhibition of antibody reaction by *M. phlei* preabsorption was observed with 44 serum samples from two other dairy herds with suspected serological false-positive reactions (data not shown), indicating that the observed antibody binding inhibition with *M. phlei* may not be a herd-specific phenomenon. The reason for this difference is presently unknown. The antigens used in EVELISA were prepared by gently extracting extracellular molecules using 80% ethanol (Eda et al., 2006), suggesting that the antigen is composed of molecules associated loosely with the surface of MAP. Although the information about the antigens used in ELISA-A is not available, it is plausible that protoplasmic antigens (PPA) were used as described in previous papers (Sockett et al., 1992; Klausen et al., 2003; Paolicchi et al., 2003). PPA is a crude antigen mixture prepared by thorough disruption of mycobacterial bacilli followed by removal of cell debris and cell wall components (Beam et al., 1969). Considering the process of PPA preparation, it is likely that the preparation contains intracellular proteins that are well conserved among mycobacterial species (Bannantine et al., 2002; Li et al., 2005). Therefore, a possible explanation for the difference in the effect of *M. phlei* on EVA-ELISA and ELISA-A results is that antigens used in the ELISA-A contained conserved proteins that cross-reacted with the false-positive sera, whereas EVA-ELISA antigens did not.

**Conclusions**

Assuming the sensitivity of ELISA-A is at most 70% (in this study, its sensitivity in fecal culture-positive animals was much lower at 48.5%), the sensitivity of the EVA-ELISA when applied to fecal culture-negative animals was shown to be higher than that of the ELISA-A using a Bayesian analysis (Branscum et al., 2005). This study also indicates that the specificity of ELISA test for JD may be improved by using the EVA-ELISA, especially in herds where current ELISA tests show low specificity.

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Disclosure Statement

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